10.1161/CIRCULATIONAHA.121.058489

Skeletal Muscle Disorders: A Non-cardiac Source of Cardiac Troponin T

Running Title: *Mueller et al.; Cardiac Troponins and Skeletal Muscle Disease*

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Abstract

Background: Cardiac troponin T (cTnT) and cTnI are considered cardiac-specific and equivalent in the diagnosis of acute myocardial infarction. Previous studies suggested rare skeletal myopathies as a non-cardiac source of cTnT. We aimed to confirm the reliability/cardiac specificity of cTnT in patients with various skeletal muscle disorders (SMD).
Methods: We prospectively enrolled patients presenting with muscular complaints (≥2 weeks) for elective evaluation in four hospitals in two countries. After cardiac work-up, patients were adjudicated into three predefined cardiac disease categories. Concentrations of cTnT/I and resulting cTnT/I mismatches were assessed using high-sensitivity cTnT (hs-cTnT-Elecsys) and three hs-cTnI assays (hs-cTnI-Architect, hs-cTnI-Access, hs-cTnI-Vista), and compared to controls without SMD presenting with adjudicated non-cardiac chest pain to the emergency department (n=3508, mean age 55y, 37% female). In patients with available skeletal muscle biopsies, TNNT/I1-3 mRNA differential gene expression was compared to biopsies obtained in controls without SMD.
Results: Among 211 patients (mean age 57y, 42% female), 108 (51%) were adjudicated to having no cardiac disease, 44 (21%) mild and 59 (28%) severe cardiac disease. hs-cTnT/I concentrations significantly increased from patients with no versus mild versus severe cardiac disease for all assays (all p<0.001). hs-cTnT-Elecsys concentrations were significantly higher in patients with SMD versus controls (median 16ng/L (IQR 7-32.5) versus 5ng/L (IQR 3-9), p<0.001) while hs-cTnI concentrations were mostly similar (hs-cTnI-Architect 2.5ng/L (IQR 1.2-6.2) versus 2.9ng/L (IQR 1.8-5.0), hs-cTnI-Access 3.3ng/L (IQR 2.4-6.1) versus 2.7ng/L (IQR 1.6-5.0) and hs-cTnI-Vista 7.4ng/L (IQR 5.2-13.4) versus 7.5ng/L (IQR 6-10)). hs-cTnT-Elecsys concentrations were above the upper-limit of normal (ULN) in 55% of patients with SMD vs 13% of controls (p<0.01). mRNA analyses in skeletal muscle biopsies (n=33), mostly (n=24) from non-inflammatory myopathy and myositis, showed 8-fold upregulation of TNNT2, encoding cTnT (but none for TNNI3, encoding cTnI); versus controls (n=16, pWald <0.001), the expression correlated with pathological disease activity (R=0.59, pt-statistic <0.001) and circulating hs-cTnT concentrations (R=0.26, pt-statistic =0.031).
Conclusion: In patients with active chronic SMD, elevations in cTnT concentrations are common and not due to cardiac disease in the majority. This was not observed for cTnI, and may in part be explained by re-expression of cTnT in skeletal muscle.

Clinical Trial Registration: URL: https://www.clinicaltrials.gov; Unique identifier: NCT03660969.

Key Words: High-sensitivity; Troponin; Myocardial Infarction; Myopathy; Skeletal

Non-standard Abbreviations and Acronyms: AMI, Acute myocardial infarction; CAD, Coronary artery disease; CK, Creatine kinase; CMR, cardiac magnetic resonance imaging; cTnT, Cardiac troponin T; cTnI, Cardiac troponin I; CV, Coefficient of variation; DGE, Differential gene expression; ECG, Electrocardiogram; ED, Emergency department; ESC, European Society of Cardiology; hs-cTnT, High-sensitivity cardiac troponin T; hs-cTnI, High-sensitivity cardiac troponin I; IQR, Interquartile range; LoD, Limit of Detection; NT-proBNP, N-terminal pro-B-type natriuretic peptide; SD, Standard deviation; SMD, Skeletal muscle disease; ULN, Upper limit of normal

Clinical Perspective

What is new?

- hs-cTnT-Elecsys concentrations were above the upper-limit of normal (ULN) in >50% of patients with active chronic skeletal muscle disease and significantly higher versus controls.
- Hs-cTnI concentrations measured with three different were above the biologic-equivalent ULN in <25% of patients and comparable to controls, thereby leading to hs-cTnT/hs-cTnI mismatches in up to 50% of patients.
- hs-cTnT-Elecsys elevations in patients without cardiac disease were largely restricted to patients with active non-inflammatory myopathy and myositis.
- mRNA analyses in skeletal muscle biopsies showed 8-fold upregulation of TNNT2, encoding cTnT versus controls and the expression correlated with circulating hs-cTnT concentrations.

What are the clinical implications?

- In patients presenting with suspected AMI, the presence of active chronic non-inflammatory myopathy and myositis, should be considered as a major confounder of hs-cTnT-concentration, but not hs-cTnI-concentrations.
- These patients are at risk of erroneous AMI diagnosis with hs-cTn, where hs-cTnI is the preferred analyte.
- In patients with other skeletal muscle disease, hs-cTnT seemed to retain cardiac specificity.

Introduction

The troponin complex is composed of three isoforms (T, I and C) and is essential for contraction of striated muscle.1,2 While the function of troponins is very similar, their amino acid sequences and configuration in cardiac and skeletal muscle differ.1,2 Cardiac troponins (cTn) are rapidly released when, for example, cardiomyocytes experience ischemic damage and have become a central component in the early diagnosis of acute myocardial infarction (AMI).3 The development and clinical implementation of high-sensitivity cTn (hs-cTn) assays has enabled precise discrimination of mild cTn elevations from normal cTn concentrations.4 In addition, high-sensitivity cTn-based rapid algorithms have substantially accelerated and facilitated the early diagnosis of AMI.5,6

Specificity issues with early iterations of the cTnT assays had appeared and were believed to be due to a cross-reactivity between the cTnT assay and skeletal muscle epitopes.7,8 However, falsely elevated concentrations of cTnT were considered a problem solved when using the latest version of the cTnT assay.9–15 The specificity deficit of earlier versions of the assay have been highlighted for instance by Ricchuti et al.7, who found evidence of cTnT in the skeletal muscle of patients with chronic renal disease. Given the epitopes recognized by the antibodies of the commercial cTnT assay used by Ricchuti et al. in 1998 and the variable presence of these cTnT isoforms in skeletal muscle, these same authors postulated that the modified, second-generation cTnT assay would not detect these isoforms if they are released from skeletal muscle into the circulation.7

Therefore, current clinical practice guidelines consider cTnT and cTnI cardiac-specific, equivalent, and interchangeable in the diagnosis of AMI.6 This concept has been again challenged by recent studies using the latest generation of the hs-cTnT assay showing evidence

of re-expressed cTnT in diseased skeletal muscle of patients with neuromuscular disorders, commonly resulting in cTnT-, but only rarely in cTnI-elevations in the systemic circulation.[11,12]

While previously documented, the clinical implications of these translational findings for patients presenting with skeletal muscle disorders (SMD), as well as the overall population presenting with suspected AMI are incompletely understood.[16] There are, for example, uncertainties related to the fact that out of all studies, only three used the hs-cTnT assay, previous studies often had a small sample size and mostly investigated selected patients with rare neuromuscular disorders, some with cardiac involvement that possibly contributed to systemic cTnT concentrations. Furthermore, some studies had selection bias enrolling exclusively patients with cTnT-elevations instead of consecutive unselected patients, and used only one cTnI assay as comparator.[9,11,12,17]

Therefore, we performed a prospective international multicenter study to address these uncertainties using four hs-cTnT/I assays in a broad population of patients presenting with muscular complaints.

Materials and Methods

The data, code, and study material that support the findings of this study are available from the corresponding author on reasonable request.

Study Design, Setting, and Patient Population

This is the primary analysis of the Heart & Muscle BASEL XII Study (NCT03660969), an ongoing prospective international multicenter diagnostic study enrolling patients presenting with active chronic muscular complaints for elective ambulatory or in-patient evaluation in a

neuromuscular, rheumatology, or medical service in four hospitals in two countries (Basel, Aarau and Zürich in Switzerland and Innsbruck in Austria). Active chronic muscular complaints were defined as any symptom related to muscle disease lasting for at least 2 weeks. The study was designed to contribute to a better understanding of the reliability of cTnT and cTnI for the diagnosis of AMI in the presence of SMD. Adult patients presenting with a broad spectrum of muscular complaints e.g. muscle pain, weakness (defined as scoring ≤ 4 on the Medical Research Council scale for muscle strength18), atrophy, stiffness, and fasciculations were recruited. Patients were excluded if they had acute trauma, acute medical disease such as sepsis, AMI, stroke, or other acute cardiac diseases, or terminal kidney failure requiring dialysis. The study was conducted according to the principles of the Declaration of Helsinki and approved by the local ethic committees. Written informed consent was obtained from all patients. The authors designed the study, gathered and analyzed the data according to the STROBE guidelines for observational studies (Table S1),19 vouched for the data and analysis, wrote the paper, and made the decision to submit the manuscript for publication. Data was entered in a dedicated RedCap database20.

Clinical Assessment

Work-up for skeletal muscle disease was performed according to local standard operating procedures. The diagnosis of SMD was established by the treating clinicians (neurologists, rheumatologists and internists) at the recruiting centers. All patients went through a thorough muscular and neurologic diagnostic process including laboratory testing (such as antibody screens), chest imaging, electro(neuro)myography, muscle MRIs, genetic testing and muscle biopsies analyses, as clinically indicated. After work-up was completed, final diagnoses were adjudicated in conjunction with a neurologist into six groups: non-inflammatory myopathies,

neuropathies, myasthenic syndromes, myositis (primary, secondary and overlap syndromes), auto-immune diseases with muscular symptoms and muscle symptoms of unknown origin (Table S2). Non-inflammatory myopathies included myotonic dystrophy, facioscapulohumeral muscular dystrophy, limb–girdle muscular dystrophy, mitochondrial disease and glycogen storage disease. Myositis included dermatomyositis, polymyositis, sporadic inclusion body myositis, hereditary inclusion body myositis, Immune mediated necrotizing myositis, myositis with overlap with collagenous disease, statin-induced myositis and vasculitis.

Standardized cardiac assessment included a structured questionnaire for history of cardiovascular disease and cardiovascular risk factors, physical examination, 12-lead electrocardiogram (ECG), measurement of N-terminal pro-B-type natriuretic peptide (NT-proBNP) as a quantitative marker of hemodynamic cardiac stress21, cTnT, cTnI, and cardiac imaging including echocardiography and cardiac magnetic resonance imaging (CMR) whenever indicated by clinical uncertainty regarding the underlying cardiac disease including cTnT/I elevations.

Classification According to Cardiac Disease

Using predefined criteria, patients were centrally adjudicated into three categories according to the presence and extent of cardiac disease (no cardiac disease, mild cardiac disease, severe cardiac disease) by investigators blinded to cTnT/I results. According to current guidelines6, cTnT and cTnI are quantitative markers of ongoing cardiomyocyte damage, elevations in cTnT or cTnI as a reflection of true cardiac disease may be present in a relevant proportion of patients with severe cardiac disease, are unlikely in patients with mild cardiac disease, and very unlikely in patients with no cardiac disease.

Patients with prior AMI, chronic heart failure (NYHA class ≥ II), severe valvular disease, left ventricular ejection fraction below 40%, CMR showing late gadolinium enhancement, or NT-proBNP concentrations above 400 ng/L21 were classified as having severe cardiac disease. In the absence of these criteria, patients with coronary artery disease (CAD), atrial fibrillation/flutter, left or right bundle branch block on ECG, left ventricular hypertrophy, mild-to-moderate valvular disease or any other structural or functional abnormality (abnormal motility, dilation) detected in echocardiography or CMR, or NT-proBNP concentrations 125-400 ng/L21 were classified as having mild cardiac disease. All other patients were classified as having no cardiac disease.

Endpoints

The primary clinical endpoints were systemic hs-cTnT/I concentrations, the prevalence of cTnT/I elevations in the overall cohort as well as in patients with no cardiac disease, and the resulting cTnT/I mismatches. Secondary endpoints were the patient-specific correlation of cTnT/I and creatine kinase (CK) as a quantitative marker of skeletal muscle injury. The translational endpoints for mRNA analyses were the gene expression in the six TnT/I genes in cases versus controls and the correlation of the three TnT genes with circulating hs-cTnT concentrations among cases.

Control Cohorts

hs-cTnT/I concentrations and the prevalence of hs-cTnT/I mismatches were compared to a control cohort of patients presenting to the emergency department (ED) with acute chest pain and adjudicated non-cardiac cause (n=3508, mean age 55 years, 37% women, Table S3) within an

international multicenter study (APACE, ClinicalTrials.gov registry number NCT00470587, Supplemental methods).

mRNA analyses were compared to a control cohort of consecutive patients free from known SMD undergoing hip replacement surgery at the University Hospital of Basel, who were asked for consent to perform an intra-operative skeletal muscle biopsy (n=16, mean age 68 years, 44% women). No other exclusion criteria applied. Thus, patients had cardiac and non-cardiac comorbidities. Skeletal muscle tissue samples were processed by the Pathology Department of the University of Basel similarly to the skeletal muscle tissue samples obtained from patients with SMD to allow for mRNA extraction and analysis.

No matching was performed between the SMD patients and patients from the control cohorts.

Laboratory Measurements

One set of venous blood samples were drawn at enrolment via a peripheral intravenous line and heparin plasma was then immediately processed for the measurement using the most widely applied high-sensitivity cTnT assay (Roche hs-cTnT-Elecsys) and Abbott-hs-cTnI-Architect assays, or frozen at -80°C until assayed for measurement using Siemens-hs-cTnI-Dimension Vista or Beckman-hs-cTnI-Access assays. Additionally, plasma CK, CK-MB isoenzyme as measured by immunoassay and plasma creatinine (Cobas automated analyzer, Roche Diagnostics) and NT-proBNP (Elecsys proBNP assay, Roche Diagnostics22) were measured. The hs-cTnT-Elecsys assay (Elecsys 2010 hs-cTnT, Roche Diagnostics) has a 99th-percentile concentration (upper limit of normal, ULN) of 14 ng/L with a coefficient of variation (CV) of 10% at 13 ng/L. Limit of blank and limit of detection (LoD) have been determined to be 3 ng/L

and 5 ng/L. Sex-specific ULNs were determined at 15.5 ng/L for men and 9 ng/L for women.23 The hs-cTnI-Architect assay (ARCHITECT STAT high-sensitivity troponin I, Abbott Laboratories) has a 99th-percentile concentration of 26 ng/L with a CV of < 5%, a limit of blank of 1.3 ng/L and a LoD of 1.9 ng/L. Sex-specific ULNs were defined at 34.2 ng/L for men and 15.6 ng/L for women.24,25

The hs-cTnI-Access assay (ACCESS hs-cTnI, Beckman Coulter) has an overall 99th-percentile concentration of 18.2 ng/L with a CV of < 10%. Limit of blank and LoD have been determined to be 1.7 ng/L and 2.3 ng/L, respectively. Sex-specific ULNs were defined at 20.9ng/L for men and 9.6ng/L for women.26 The hs-cTnI-Dimension Vista has an overall 99th-percentile concentration of 58.9 ng/L with a CV of < 5%, a limit of blank of 1ng/L and a LoD of 2 ng/L. Sex-specific ULNs were defined at 68ng/L for men and 44ng/L for women.27 The uniform and sex-specific ULNs used in the current analysis are consistent with the ULNs provided by the IFCC Committee on Clinical Applications of Cardiac Biomarkers.28

The different hs-cTnI assays examined use different antibody combinations, are not standardized and thus the ratios of T/I will differ between assay.29 Therefore, we expected varying rates of hs-cTnT/I mismatches depending on the hs-cTnI assay used.

mRNA Analysis

Muscle tissue samples were obtained during patient work-up whenever clinically indicated. RNA extraction was performed by CEGAT (Tübingen, Germany) using the RNeasy Mini Kit (Qiagen, Netherlands) or RNeasy Fibrous Tissue Mini kit (Qiagen, Netherlands) after sample randomization. Libraries were prepared and RNA sequenced as detailed in the supplemental. After mapping, pre-processing and quality control, differential gene expression (DGE) analysis

was conducted on all sequenced samples using the summarized gene counts with DESeq2 v1.28.0.30 The DGE analysis of cases was compared to controls. In total, six troponin genes were tested for DGE (TNNT2, TnT cardiac type, TNNT1, TnT slow skeletal type, TNNT3, TnT fast skeletal type, TNNI3, TnI cardiac type, TNNI1, TnI slow skeletal type, TNNI2, TnI fast skeletal type). Importantly, the entire process of RNA extraction and analysis was conducted blinded to the blood concentrations of hs-cTnT/I for all cases and controls. To correlate TNNT2 expression with disease activity, a score was derived based on 18 marker variables resulting from visual biopsies analysis by specialized pathologists at the respective centers. (Supplemental methods, Figure S1 and S2).

Determination of Sample Size

Based on prior literature,11,12 we estimated the proportion of patients to present an elevated hs-cTnT in the overall cohort to be 67% and the proportion of patients presenting an elevated hs-cTnI to be 10%. Based on initial preliminary data, we conservatively predicted the proportion of patients without cardiac disease to be 25% in our cohort. We estimated at 10% of the patients lacking at least one of the three hs-cTnI measurement. For a selected power of 90% and a two-sided type I error of 0.05 which was then adjusted for multiple testing (three comparison with each hs-cTnI assay), a minimal sample size of 176 patients was calculated to detect a difference in proportion of hs-cTn between the two assays using a McNemar test and to allow sufficient power the population with no cardiac disease. Further details to the derivation of the sample size are given in the supplemental.

Statistical Analysis

As recent studies have reported that approved uniform 99th-percentiles ULN are not biologically equivalent,31 biologically equivalent ULN derived from a large prospective diagnostic study with parallel measurement of the respective hs-cTnT/I assays was used for the primary analysis (Supplemental methods, Figures S3 and S4) for the hs-cTnI-Architect and hs-cTnI-Access assays. In brief, the biologically equivalent ULNs for hs-cTnI-Architect and hs-cTnI-Access to the ULN of 14 ng/L for hs-cTnT were derived in APACE32 and found to be 6.6 ng/L and 6.9 ng/L, respectively31,33. For the hs-cTnI-Vista assay, the biologically equivalent ULN of 42 ng/L to the ULN of 14 ng/L for hs-cTnT was derived in a recent study of healthy individuals.27 Secondary analyses used the manufacturer-recommended and regulatory authorities approved 99th-percentiles ULN described above as well as sex-specific cut-offs.25,26,31,34,35 To further assess the clinical implications for the early diagnosis of AMI in patients presenting with acute chest pain, the proportion of patients with hs-cTnT elevations above the rule-in cut-off of the European Society of Cardiology (ESC) 0/1h-algorithm (52 ng/L) was calculated.5,6,33

Continuous variables are presented as mean ± standard deviation (SD) when normally distributed and median with interquartile ranges (IQR) when non-normally distributed. Categorical variables are expressed as numbers and percentages. Independent t-tests, Mann-Whitney-U or Kruskall Wallis tests were applied for comparison of continuous variables and the Fisher's exact test was used for the comparison of categorical variables. Comparisons of proportions were made using a 2-sample test for equality of proportions with continuity correction.

A subgroup analysis was performed to evaluate whether the prevalence of cTnT/I elevations differed among the different underlying etiologies of muscular complaints. When hs-

cTnT/I concentrations were used in statistical modelling or analyses, concentrations were transformed on the logarithmic scale to approximate normal distribution.

Correlations were assessed using the Kendall rank correlation coefficient, the coefficient of determination (R-squared) and p-values provided by linear regressions. The Benjamini and Hochberg (BH) method was used to correct for multiple testing where appropriate36. Statistical analyses were performed using the R statistical package (Vienna, Austria).

For mRNA analyses, resulting P-values attained by the Wald test were also corrected for multiple testing using the BH method. An adjusted P-value <0.05 was considered significant. A subgroup analysis on the major gene of interest, TNNT2 (cardiac type) was conducted by correlating a SMD activity score with TNNT2 gene expression while correcting for SMD etiology (myopathy, myositis or other SMD).

Results

Patient Characteristics and Assessment of Cardiac Health

From August 2018 to October 2020, 223 patients were enrolled and 211 patients were eligible for this analysis (Figure S5). The mean age was 57 years, 88 (42%) were women, 23 (11%) had known CAD, 16 (8%) prior AMI, 15 (7%) a history of atrial fibrillation, 81 (38%) arterial hypertension, and 33 (16%) diabetes mellitus (Table 1). Patients were mainly recruited during ambulatory evaluation of their muscle disorder (188, 89%). Most patients presented with muscle weakness (n=129, 61%) and/or muscle pain (n=87, 41%). Functional limitations such as dysphagia, dyspnea, incontinence, digestive symptoms or falls were present in 2 to 14% of the patients. Echocardiography was performed in 56% of the patients and CMR in 22% (Table S4).

Non-inflammatory myopathy, myositis, and myasthenic syndrome were the most common final diagnosis after work-up (Table S2). Cardiac characterization classified 59 patients (28%) as having severe cardiac disease, 44 (21%) mild cardiac disease, and 108 patients (51%) no cardiac disease (Table 2).

hs-cTnT/I Concentration and hs-cTnT/I Mismatches

hs-cTnT/I concentrations increased significantly from patients with no cardiac disease versus patients with mild cardiac disease versus patients with severe cardiac disease for all assays (all pMann-Whitney<0.001). In the overall group, hs-cTnT-Elecsys concentrations were above the uniform approved ULN in 55%, and significantly higher versus controls (median 16ng/L (IQR 7-32.5) versus 5ng/L (IQR 3-9), pMann-Whitney<0.001, Figure 1, Table S5). Elevations in hs-cTnT were even above the rule-in cut-off of the ESC 0/1h-algorithm and of the ESC 0/2h-algorithm (52ng/L) in 34 patients (16.1%, Table S6). In contrast, hs-cTnI-Architect, hs-cTnI-Access, and hs-cTnI-Vista concentrations were above the biologic-equivalent ULN in 23%, 23%, and 8%, and overall comparable to controls (hs-cTnI-Architect 2.5ng/L (IQR 1.2-6.2) versus 2.9ng/L (IQR 1.8-5.0), hs-cTnI-Access 3.3ng/L (IQR 2.4-6.1) versus 2.7ng/L (IQR 1.6-5.0) and hs-cTnI-Vista 7.4ng/L (IQR 5.2-13.4) versus 7.5ng/L (IQR 6-10), Table S5). This resulted in hs-cTnT/hs-cTnI-mismatches in 36-50% in the overall cohort and in 33-37% in patients without cardiac disease (Figure 2, Table S7). These findings were confirmed using uniform approved and sex-specific ULN (Supplemental Figures 6-9). In the control cohort, hs-cTnT/hs-cTnI mismatches were uncommon (4 to 5% using biologically equivalent ULN, Figure S10, Table S8).

Impact of Underlying Etiology on CK and hs-cTnT/I Concentrations

When the different etiologies of muscle disorders were analyzed separately, relevant differences among the etiologies emerged in CK and hs-cTnT concentrations, which were not observed with the hs-cTnI Architect, hs-cTnI-Access, and hs-cTnI-Vista assays (Figure 3A). Non-inflammatory myopathies and myositis had the highest CK and hs-cTnT concentrations, both in the overall cohort and in the subgroup of patients without cardiac disease (Figure 3B). hs-cTnT elevations in patients without cardiac disease were largely restricted to patients with non-inflammatory myopathies and myositis. Within this subgroup of non-inflammatory myopathies and myositis, 77% (in the overall cohort) and 68% (in the subgroup with no cardiac disease) presented hs-cTnT concentrations > uniform approved ULN, while only a few of these patients also showed a hs-cTnI elevation (pz-test<0.001, Table S9; Figure 3, Figures S11 and S12). In contrast, the vast majority of patients with neuropathies, myasthenic syndromes and auto-immune diseases had normal hs-cTnT concentrations.

Correlation between hs-cTnT/I and CK as Quantitative Indicator of Muscle Damage

In the overall cohort, hs-cTnI concentrations did not correlate with CK or CK-MB concentrations, while hs-cTnT concentrations showed a positive significant correlation with CK and CK-MB concentrations (e.g. with CK R=0.33, and R=0.43 in subgroup of patients without cardiac disease, both ptau-statistic<0.001, Figure 4A,B; Figure S13, Table S10 and S11).

Muscle Tissue mRNA Analysis

Muscle biopsies from diseased skeletal muscle were available for 33 patients, mean age 59 years, 39% women (Table S12). DGE analysis showed significant upregulation of the gene TNNT2 coding for cTnT (Top 96 differentially expressed gene, 8-fold change as compared with controls,

pt-statistic<0.001), fast skeletal muscle TNNT3 and TNNI2 genes (Figure 5A and B). Both the cardiac TNNT2 and TNNI3 genes were expressed at low levels in control muscle biopsies, but the level of expression stayed largely below the expression of skeletal muscle troponin genes (Figure 5C). There was a significant positive correlation between the TNNT2 gene and a SMD activity score based on pathological features of the diseased skeletal muscle biopsies, independently of disease etiology, with higher disease activity score showing higher TNNT2 upregulation (R=0.59, pt-statistic<0.001, Figure 5D).

Correlation between Normalized Count of TnT Gene Expression and Circulating hs-cTnT

Among the 33 patients providing muscle tissue, circulating hs-cTnT concentrations significantly positively correlated with normalized TNNT2 (R=0.26, pt-statistic =0.032, Figure 6, Table S15) expression, but not with TNNT1 or TNNT3.

Discussion

This prospective multicenter study evaluated cTnT and cTnI concentrations using four widely applied hs-cTnT/I assays in a broad population of patients presenting with skeletal muscle symptoms. Those were compared to a large control cohort of patients adjudicated to have non-cardiac causes of acute chest pain, to assess possible implications for the diagnosis of AMI or other cardiac diseases, and cardiovascular risk-stratification. We report eight major findings.

First, about 50% of patients with SMD had mild or severe cardiac disease. Most of the cardiac abnormalities were related to common cardiac disorders such as CAD, which are associated with an increased risk of future AMI, further documenting the clinical relevance of reliable AMI diagnosis in this population. Second, hs-cTnT/I serum concentrations significantly increased from patients with no cardiac disease versus mild cardiac disease versus severe cardiac disease for all assays. Accordingly, cardiomyocyte injury due to cardiac disease was a major

contributor to hs-cTnI and hs-cTnT concentrations also in patients with SMD. Third, hs-cTnT-Elecsys concentrations were above the uniform approved ULN in 55% and significantly higher versus controls (median 16ng/L versus 5ng/L, p<0.001). In contrast, hs-cTnI-Architect, hs-cTnI-Access and hs-cTnI-Vista concentrations were above the biologic-equivalent ULN in 23%, 23%, and 8%, and overall comparable to controls. These findings were confirmed using uniform approved and sex-specific ULN. Fourth, elevations in hs-cTnT/I concentrations were most often mild. However, 16.1% of patients in the overall cohort and 12.9% in the subgroup without cardiac disease had hs-cTnT concentrations above the rule-in cut-off of the ESC 0/1h-algorithm and ESC 0/2h-algorithm (52 ng/L).5,6,33 Therefore, the proportion of SMD patients possibly misclassified by the rule-in cut-off of the ESC 0/1h-algorithm or ESC 0/2h-algorithm seemed even higher compared to more common populations with increased baseline hs-cTnT/I concentrations such as patients with renal dysfunction and the elderly.37,38 Fifth, hs-cTnT elevations in patients without cardiac disease were largely restricted to patients with non-inflammatory myopathy and myositis, whereas the vast majority of patients with neuropathies, myasthenic syndromes, auto-immune diseases, or other causes of skeletal muscle symptoms had hs-cTnT concentrations within the normal range. Sixth, hs-cTnT, but not hs-cTnI, showed a significant positive correlation with CK, a biomarker of skeletal muscle damage, providing further support for the concept that damaged skeletal muscle is the origin of some of the systemic hs-cTnT concentration.9,11,12 Seventh, in the subgroup of patients with skeletal muscle biopsies available, mRNA analyses in diseased skeletal muscle showed 8-fold upregulation of TNNT2, encoding cTnT, versus controls without SMD undergoing hip replacement. The expression strongly correlated with pathological disease activity, thereby suggesting active chronic SMD as a significant contributor to the systemic hs-cTnT concentration. This assumption was further

strengthened by a positive and significant correlation between TNNT2 gene expression and circulating hs-cTnT concentrations. Eight, in contrast, no evidence of upregulation/re-expression in diseased skeletal muscle was found for cTnI.

These findings extend and corroborate results from prior studies, including three studies using the hs-cTnT assay.9,12,17 Among 27 ambulatory patients with skeletal myopathies and muscle dystrophies, hs-cTnT concentrations were elevated in 18 patients (67%), with a median of 21 ng/l (IQR 11 to 38 ng/l), while cTnI was elevated in only one patient (4%).11 Among 74 patients with hereditary and acquired skeletal myopathies, hs-cTnT concentrations were elevated in 69%, with a median of 24 ng/l (IQR 11 to 54 ng/l), while hs-cTnI was elevated in 4% of patients.12 In 122 patients with Pompe disease, hs-cTnT concentrations were elevated in 82% of patients (median 27 ng/L), while hs-cTnI concentrations were normal in all patients. All three studies found elevated systemic concentration of hs-cTnT, but not hs-cTnI, and, in part also, evidence for some RNA and/or protein expression on skeletal muscular tissue level.

Based on these consistent findings, the following insights emerge: first, in the presence of active chronic SMD from two categories, non-inflammatory myopathy and myositis, including statin-induced myopathy, hs-cTnT loses cardiac specificity as diseased skeletal muscle contributes to the systemic hs-cTnT concentration. Second, based on mRNA analysis, re-expression of cTnT during chronic repair mechanisms in the diseased skeletal muscle appears to be the underlying pathophysiology.7,9–13,39,40 Third, re-expression of cTnT during skeletal muscle repair mechanisms seems to be time-dependent. It was present in this study of patients with active chronic SMD with ongoing skeletal muscle damage and repair lasting for weeks to months. In contrast, no evidence was found in patients with acute rhabdomyolysis, an in vivo model of acute skeletal muscle damage of several days duration, as no correlation was observed

between hs-cTnT and CK concentrations. Accordingly, hs-cTnT/I mismatches were uncommon in acute rhabdomyolysis.41,42 Fourth, other SMD categories including neuropathies, myasthenic syndromes, auto-immune diseases, or other causes of skeletal muscle symptoms do not seem to be relevant sources of systemic hs-cTnT concentration. Fourth, while a small number of patients with SMD also showed elevations in hs-cTnI, the absence of an increase in cTnI mRNA at the tissue level and the absence of correlation with CK highlighted both in this and previous studies clearly argue against a skeletal muscle origin.7,17 Alternative explanations need to be considered in these patients, such as analytical interference e.g. due to heterophilic antibodies, autoantibodies, or the formation of macro-troponin complexes, which seem to be affect hs-cTnI more commonly than hs-cTnT.43–47 The interpretation of CK-MB is difficult, as it has cardiac and skeletal muscle sources and is re-expressed in diseased skeletal muscle.3

Contrary to what was expected, the prevalence of hs-cTnT/I mismatch was higher in the overall group with about half having documented cardiac disease versus the subgroup without cardiac disease (36-50% versus 33-37%). This indicates that to some degree preferential release of cTnT versus cTnI from cardiomyocytes due to chronic cardiac disease may have also contributed to hs-cTnT/I mismatch. The exact pathophysiology underlying this differential release is largely unknown.48 Alternatively, differences in renal function between the overall group and patients with no cardiac disease could have led to differences in clearance between the hs-cTnT and hs-cTnI circulating concentrations.

These findings have clinical implications. In patients presenting with suspected AMI and without ST-segment elevation, the presence of active chronic non-inflammatory myopathy or myositis as a possible important confounder of hs-cTnT concentrations must be actively assessed in institutions using hs-cTnT as their standard of care in the ED, as the risk of erroneous AMI

diagnosis is increased in these patients. If no SMD or SMD other than these two categories are present, no change in their standard of care seems necessary. If patient history reveals active chronic non-inflammatory myopathy or myositis, hs-cTnI rather than hs-cTnT should be measured as an alternative, if available. If hs-cTnI is not available, resampling at 1h or 2h would be mandatory to differentiate AMI with its rise within 1h or 2h versus non-cardiac causes of chest pain with usually stable hs-cTnT concentrations5,6,33. A similar change in management should also be considered in other acute disorders, in which an elevated hs-cTnT concentration is associated with a change in management, such as for instance rhythm monitoring and/or escalation of therapy as in peri-/myocarditis and in patients with acute pulmonary embolism. Although the prevalence of patients with active chronic non-inflammatory myopathy or myositis in previous diagnostic studies deriving rapid hs-cTnI-based triage algorithms likely was very small, our findings provide further support for the more sophisticated methodology of using two adjudicated final diagnoses: one using serial measurements of hs-cTnT, and one of hs-cTnI.49,50 Finally, our results highlight the need for future hs-cTnT assays to ensure that their antibodies do not cross react to the troponin T form found in diseased skeletal muscle.

Several limitations of the present study merit consideration. First, while being the largest study performed to date, the sample size of some etiologies was only modest. Second, this study included three widely applied hs-cTnI assays. Although the findings were quite consistent among the different hs-cTnI assays, studies including other clinically used hs-cTnI assays seem warranted to explore their reliability in patients with SMD. Given the relevant differences in the antibody combination used in these different immunoassays27,35, different findings may emerge. Third, we may have misclassified a small number of patients as having no cardiac disease, as symptoms, signs, ECG, and NT-proBNP concentrations were available in all of these

patients, but cardiac imaging only in a subset. Fourth, over the span of their lifetime, 10-20% of patients with non-inflammatory myopathies and myositis seem to develop clinically apparent cardiac involvement(51–53). Therefore, in a small proportion of patients with these underlying etiologies, despite normal findings in cardiac imaging, subtle microscopic cardiomyocyte injury may have already been present and contributed to the high prevalence of hs-cTnT elevation. Future studies including long-term follow-up are necessary to provide an additional domain assessing the biological significance of elevated hs-cTnT concentrations in these patients. Fifth, it is impossible to precisely quantify the proportion of the systemic hs-cTnT concentration that was contributed by the diseased skeletal muscle versus cardiomyocyte injury in the two affected SMD categories. The modest correlation between TNNT2 gene expression and circulating hs-cTnT concentrations and the persistent association between the extent of cardiac disease and hs-cTnT concentration suggest that cardiomyocyte injury remained the dominant source. Sixth, due to the absence of serial assessments, we cannot comment on the exact timepoint at which cTnT start to be re-expressed in non-inflammatory myopathies and myositis. Longitudinal studies are required to quantify the time to re-expression.

Conclusions

In conclusion, hs-cTnT elevations are common in patients with active chronic non-inflammatory myopathy and myositis, but not with other SMD, and in part due to upregulation and thereby re-expression of TNNT2 in diseased skeletal muscle. In contrast, no evidence of upregulation/re-expression in diseased skeletal muscle was found for cTnI. Therefore, in patients with active chronic non-inflammatory myopathy and myositis, cTnI is the preferred analyte for assessing cardiac health in general and the presence of AMI.

Sources of Funding

The study was supported by research grants from the Swiss Heart Foundation, University of Basel, University Hospital of Basel, and Abbott, Roche, and Siemens.

Disclosures

We disclose that Dr. Jeanne du Fay de Lavallaz has received research support from the Swiss Heart Foundation. We disclose that Dr. Nestelberger has received research support from the Swiss National Science Foundation (P400PM_191037/1), the Prof. Dr. Max Cloëtta Foundation, the Margarete und Walter Lichtenstein-Stiftung (3MS1038), and the University Hospital Basel as well as speaker honoraria/consulting honoraria from Siemens, Beckman Coulter, Bayer, Ortho Clinical Diagnostics and Orion Pharma, outside the submitted work. Dr. Boeddinghaus has received research grants from the University of Basel and the Division of Internal Medicine, the Swiss Academy of Medical Sciences, the Gottfried and Julia Bangerter-Rhyner-Foundation, and speaker honoraria from Siemens, Roche, Ortho Clinical Diagnostics, and Quidel Corporation, outside the submitted work. Dr. Mueller has received research support from the Swiss National Science Foundation, the Swiss Heart Foundation, the KTI, the University Hospital Basel, the University of Basel, Abbott, Beckman Coulter, Idorsia, Novartis, Ortho Clinical Diagnostics, Quidel, Roche, Siemens, as well as speaker honoraria/consulting honoraria from Amgen, Astra Zeneca, Bayer, Boehringer Ingelheim, BMS, Daiichi Sankyo, Idorsia, Novartis, Osler, Roche, and Sanofi, outside the submitted work. Dr. Maurer has grant/research support from Prof. Max Cloetta Foundation, AbbVie, Protagen, Novartis Biomedical Research, received speaker fees from Boehringer-Ingelheim as well as congress support from Pfizer, Roche, Actelion, Mepha, and MSD. In addition, Dr. Maurer has a patent mir-29 for the treatment of systemic sclerosis issued (US8247389, EP2331143), all outside the submitted work. Dr. Gualandro received

research grants from FAPESP (Fundacao de Amparo a Pesquisa do Estado de Sao Paulo, Brasil) and consulting honoraria from Roche, outside the submitted work. Dr. Lopez-Ayala has received research support from the Swiss Heart Foundation (FF20079). Dr. Puelacher reports research funding from Roche Diagnostics, the University of Basel, the University Hospital Basel, outside of the submitted work. Dr. Sinnreich has received financial support from Roche from 2015 to 2019 for a research collaboration unrelated to the current work. All other authors declare that they have no conflict of interest with this study. The hs-cTn assays investigated were donated by the manufacturers, who had no role in the design of the study, the analysis of the data, the preparation of the manuscript, or the decision to submit the manuscript for publication.

Appendix

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Supplemental Material List

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Table 1.

Variable	Overall cohort	Severe cardiac disease	Mild cardiac disease	No cardiac disease	p
n	211	59	44	108	
Sex : Female (%)	88 (42)	22 (37)	17 (39)	49 (45)	0.566
Age (mean (SD))	56.8 (17.4)	66.6 (15.2)	63.2 (14.9)	48.9 (15.7)	<0.001
Hospitalized (%)	23 (11)	11 (19)	2 (5)	10 (9)	0.063
Coronary artery disease (%)	23 (11)	16 (27)	7 (16)	0 (0)	<0.001
Previous AMI (%)	16 (8)	16 (27)	0 (0)	0 (0)	<0.001
Hypertension (%)	81 (38)	34 (58)	23 (52)	24 (22)	<0.001
Hypercholesterolemia (%)	49 (23)	24 (41)	12 (27)	13 (12)	<0.001
Diabetes Mellitus (%)	33 (16)	15 (25)	8 (18)	10 (9)	0.018
History of atrial fibrillation (%)	15 (7)	14 (24)	1 (2)	0 (0)	<0.001
Previous DVT or PE (%)	11 (5)	4 (7)	4 (9)	3 (3)	0.225
Heart failure (%)					<0.001
None	192 (93)	44 (77)	42 (98)	106 (100)	
NYHA I	5 (2)	4 (7)	1 (2)	0 (0)	
NYHA II	5 (2)	5 (9)	0 (0)	0 (0)	
NYHA III	2 (1)	2 (4)	0 (0)	0 (0)	
NYHA IV	2 (1)	2 (4)	0 (0)	0 (0)	
Chronic kidney disease (%)	14 (7)	8 (14)	4 (9)	2 (2)	0.007
eGFR (median [IQR])	96.0 [76.6, 114.6]	91.8 [67.3, 107.0]	82.0 [69.2, 105.6]	102.7 [90.4, 118.1]	<0.001
Pacemaker (%)	6 (3)	4 (7)	2 (5)	0 (0)	0.014
ICD (%)	2 (1)	1 (2)	1 (2)	0 (0)	0.237
Stroke (%)	9 (4)	5 (8)	2 (5)	2 (2)	0.141
Muscle manifestations: Upper or lower body (%)					0.675

Variable	Overall cohort	Severe cardiac disease	Mild cardiac disease	No cardiac disease	p
Lower body	30 (14)	7 (12)	7 (16)	16 (15)	
Upper body	20 (9)	7 (12)	2 (5)	11 (10)	
Lower and upper body	57 (27)	15 (25)	16 (36)	26 (24)	
Not localized	104 (49)	30 (51)	19 (43)	55 (51)	
Muscle manifestations : proximal or distal (%)					0.179
proximal	10 (5)	4 (7)	3 (7)	3 (3)	
distal	39 (18)	7 (12)	6 (14)	26 (24)	
proximal and distal	58 (27)	18 (31)	16 (36)	24 (22)	
Not localized	104 (49)	30 (51)	19 (43)	55 (51)	
Begin of symptoms (%)					0.214
2-4 weeks	15 (8)	8 (15)	3 (7)	4 (4)	
>1 month, up to 12 months	43 (22)	13 (24)	9 (21)	21 (21)	
>1 year	138 (70)	34 (62)	30 (71)	74 (75)	
Muscle pain (%)	87 (41)	25 (42)	15 (34)	47 (44)	0.526
Muscle cramps (%)	25 (12)	7 (12)	7 (16)	11 (10)	0.565
Muscle atrophy (%)	54 (26)	14 (24)	11 (25)	29 (27)	0.935
Muscle stiffness (%)	19 (9)	3 (5)	4 (9)	12 (11)	0.437
Muscle weakness (%)	129 (61)	41 (69)	26 (59)	62 (57)	0.305
Skin manifestations present (%)	14 (7)	6 (10)	2 (5)	6 (6)	0.540
Joint manifestations present (%)	42 (20)	13 (22)	7 (16)	22 (21)	0.744
Clinical evaluation (%)					0.617
Planned follow-up visit	174 (82)	46 (78)	39 (89)	89 (82)	
First evaluation	30 (14)	10 (17)	5 (11)	15 (14)	
Relapse	7 (3)	3 (5)	0 (0)	4 (4)	
Symptom activity (%)					0.375
Worsening	74 (35)	23 (39)	11 (25)	40 (37)	

Variable	Overall cohort	Severe cardiac disease	Mild cardiac disease	No cardiac disease	p
Improving	15 (7)	6 (10)	3 (7)	6 (6)	
Stable	122 (58)	30 (51)	30 (68)	62 (57)	
Dysphagia (%)	29 (14)	11 (19)	3 (7)	15 (14)	0.216
Dyspnea (%)	24 (11)	9 (15)	5 (11)	10 (9)	0.509
Incontinence (%)	5 (2)	3 (5)	0 (0)	2 (2)	0.288
Digestive symptoms (%)	10 (5)	3 (5)	0 (0)	7 (7)	0.221
Falls (%)	13 (6)	3 (5)	3 (7)	7 (7)	1.000
Any cardiac medication (%)[a]*	96 (45)	45 (76)	27 (61)	24 (22)	<0.001
Final diagnosis					NA
Non-inflammatory myopathy[b]†	51 (24)	11 (19)	14 (32)	26 (24)	
Muscle symptoms	20 (9)	3 (5)	2 (5)	15 (14)	
Neuropathy	21 (10)	5 (8)	3 (7)	13 (12)	
Myasthenic syndrome	43 (20)	11 (19)	11 (25)	21 (19)	
Myositis[c]‡	53 (25)	21 (36)	7 (16)	25 (23)	
Autoimmune disease with muscle symptoms	23 (11)	8 (14)	7 (16)	8 (7)	

[a]*Cardiac medications: Any of cardiac aspirin, anti-platelet agent, beta-blocker, Angiotensin converting enzyme inhibitor or aldosteron-receptor antagonist, calcium channel antagonist, nitrates, alpha-blockers, diuretics, anti-arrhythmics or digitalis.

[b]†Non-inflammatory myopathy: Myotonic dystrophy, Facioscapulohumeral muscular dystrophy, Limb–girdle muscular dystrophy, Mitochondrial disease, Glycogen storage disease.

[c]‡Myositis: Dermatomyositis, Polymyositis, Sporadic inclusion body myositis, Hereditary inclusion body myositis, Immune mediated necrotizing myositis, Myositis with overlap with collagenous disease, Statin induced myositis, Vasculitis.

AMI=Acute myocardial infarction, DVT = Deep venous thrombosis, eGFR = estimated Glomerular filtration rate, ICD = Implantable cardiac defibrillator, NYHA = New York Heart Association, SD = Standard deviation, PE = Pulmonary embolism

estimated Glomerular filtration rate was calculated using the CKD-EPI formula

p-values comparing the three groups with different prevalence of cardiac diseases (no, mild, severe) and are derived using following tests : Chi-square tests with continuity correction for categorical variables, ANOVA for normally-distributed variables (presented with mean ± sd) and Kruskal-Wallis tests for non-normally distributed variables (presented with median [IQR])

Table 2.

Number	Overall
n	211
Cardiac disease (%)	
No cardiac disease	108 (51)
Mild cardiac disease	44 (21)
Severe cardiac disease	59 (28)
Severe cardiac disease (%)	
Cardiomyopathie	1 (2)
History of HF (NYHA II-IV)	4 (7)
LGE	7 (12)
LVEF ≤40%	1 (2)
NTproBNP>400pg/mL	24 (41)
previous AMI	11 (19)
Mild cardiac disease (%)	
ECG: complete bundle branch block	13 (15)
ECG: LVH	2 (2)
History of atrial fibrillation	11 (13)
History of CAD	11 (13)
NTproBNP 125-400pg/mL	35 (42)
TTE or CMR: Dilation of LV or RV	1 (1)
TTE or CMR: LVH	4 (5)
TTE or CMR: Reduced Motility of LV or RV	7 (8)

AMI = Acute myocardial infarction, CAD = Coronary artery disease, CMR = Cardiovascular *magnetic resonance imaging*, ECG = electrocardiogram, HF = Heart failure, LGE = Late gadolinium enhancement, LV = Left ventricle, LVEF = Left ventricular ejection fraction, LVH = Left ventricular hypertrophy, NTproBNP = N-terminal pro-B-type natriuretic peptide, NYHA = New York Heart Association, RV = Right ventricle, TTE = Transthoracic echocardiogram.

Figure Legends

Figure 1: Violine Plots Representing the Distribution of hs-cTnT/I Concentrations for the Four Tested Assays and across Categories of Cardiac Disease. A single comparison using a Mann-Whitney-U test was conducted between the controls of the APACE cohort and the overall cohort of patients with skeletal muscle disease. Bioequivalent and overall approved Upper Limit of Normal (ULN) are represented as broken lines. hs-cTnT-Elecsys and hs-cTnI-Architect concentrations were available in all 211 patients, hs-cTnI-Access concentrations in 187 patients, and hs-cTnI-Vista concentrations in 194 patients. The p-values were calculating using a Wilcoxon-test comparing the overall group with the control group and have been corrected for multiple testing (4 tests) using the Benjamini and Hochberg method.

Figure 2: Inter-assay hs-cTnT/I Mismatches Using Biologically-equivalent Upper Limits of Normal (ULN). For each subpanel, two hs-cTnT/I assay are represented with their biologically-equivalent assay-specific 99th-percentile Upper Limit of Normal (ULN). In each panel, the four quadrants represent the percentage of patients with the following constellations: green when both hs-cTnT/I assays were below the ULN, grey when both were above the ULN, and red when there was a hs-cTnT/I mismatch (with one of the assay above and one of the assay below the ULN). A) overall cohort, B) Subgroup without cardiac disease.

Figure 3: The Different Etiologies of the Skeletal Muscle Disorders are Represented on the X-Axis and the Concentrations of the Biomarkers are Represented on the Y-axis Using a Logarithmic Scale. Boxplots represents the interquartile range (IQR) and whiskers ± 1.5*IQR. Bioequivalent and overall approved Upper Limit of Normal (ULN) are represented as broken lines.

A) in the overall cohort, B) in the cohort without cardiac disease. AD = Autoimmune disease. The p-values have been corrected for multiple testing using the Benjamini and Hochberg method.

Figure 4: Correlation between Creatine Kinase (CK) and hs-cTn in A) the Overall Cohort and in B) Patients with No Cardiac Disease. Biomarkers have been log-transformed to approximate normal distribution.

Figure 5:

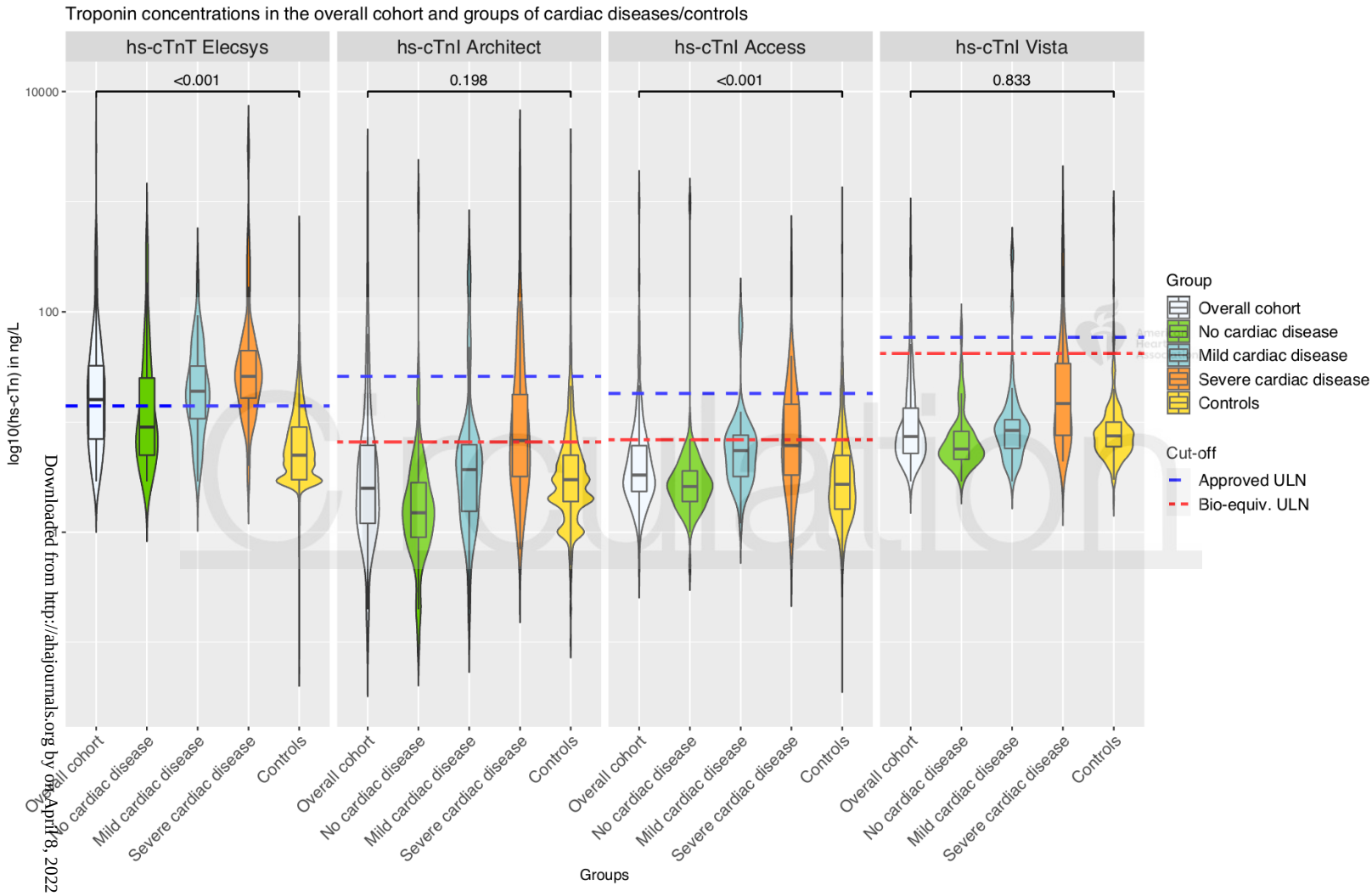
A. Differential Gene Expression (DGE) Results from a Case/Control Study Comparing Skeletal Muscle Biopsies from Patients with (33 cases) and without (16 controls) Skeletal Muscle Disease (SMD). After correcting for multiple testing using Benjamini and Hochbert, 847 of 17,124 protein coding genes were upregulated and 966 were downregulated at a significance level of $\alpha = 0.05$. Three of six genes of the Troponin gene family show a significant upregulation (TNNT2, top 96 differentially expressed gene [DEG]; TNNT3, top 881 DEG; TNNI2, top 1,821 DEG).

B. Detailed DGE Results for Six Genes of the Troponin Gene Family. Fold changes and significance levels are concordant among the slow (TNNT3 and TNNI2) and fast (TNNT1, TNNI1) skeletal muscle gene pairs but not for the cardiac gene pair (TNNT2 and TNNI3).

C. Base Level Expression of the Troponin Gene Family in Skeletal Muscle. Cardiac genes TNNT2 and TNNI3 exhibit an expression of 6 and 6.3 TPM (transcripts per million), ranking among top 39% and 38% expressed protein coding genes in the DGE analysis. Fast and slow skeletal muscle genes TNNT1, TNNI1, TNNT3, TNNI2 exhibit a mean expression of 6,848, 3,614, 1,590 and 1,418 TPM (all top 0.1%).

D. Variation of TNNT2 Expression in the Case Samples (n = 28 after Filtering for Missingness in Marker Variables for Disease Activity) Can Be Explained by Biopsy-specific Disease Activity. Linear regression shows a significant positive correlation ($R = 0.59$, $p < 0.001$) between a disease activity score derived from 14 disease activity markers and normalized counts. The score remains significant ($p = 0.001$) after adjusting for disease class ("Myopathy" [n = 7], "Myositis" [n = 13], "Other SMD" [n = 8]). Conversely, the case subset showed borderline significant differences between disease classes after adjusting for disease activity score in a likelihood ratio test ($p = 0.069$). A detailed description of the score calculation is available in the supplements.

Figure 6: Correlation between Normalized Gene Expression of the Three TnT Genes and Circulating hs-cTnT Concentrations. hs-cTnT concentrations and normalized gene expression have been log-transformed to approximate normal distribution.



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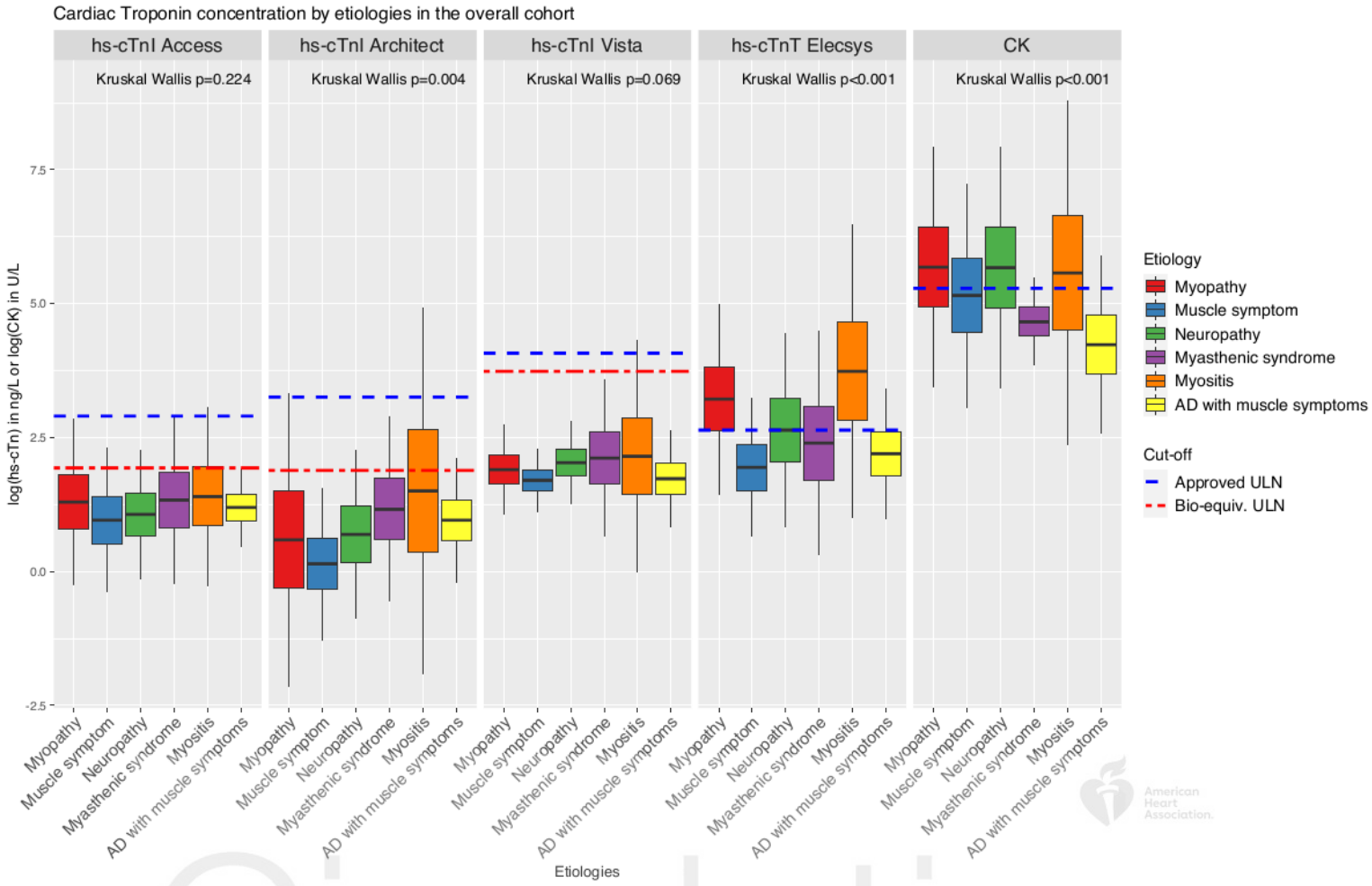

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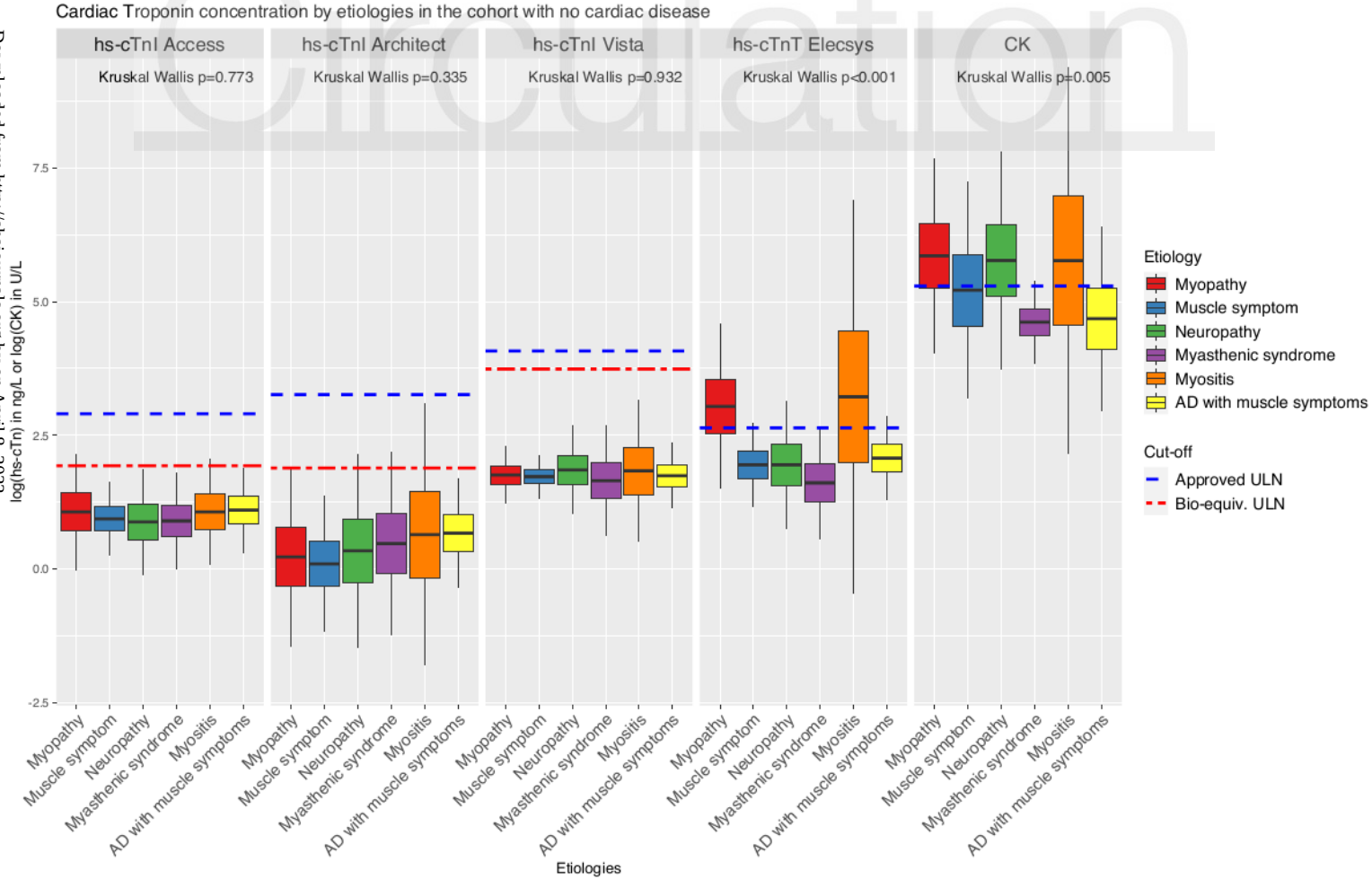

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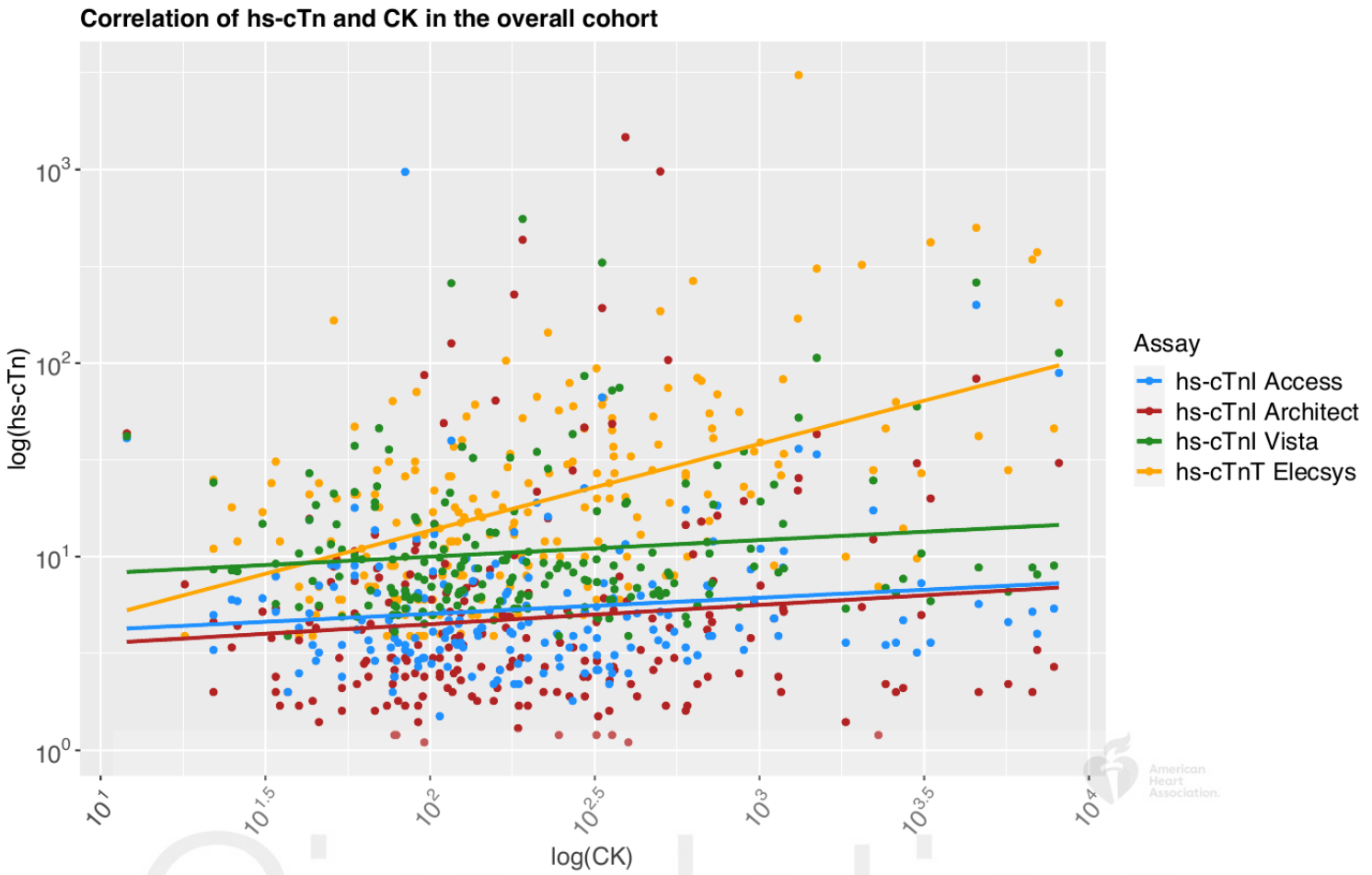

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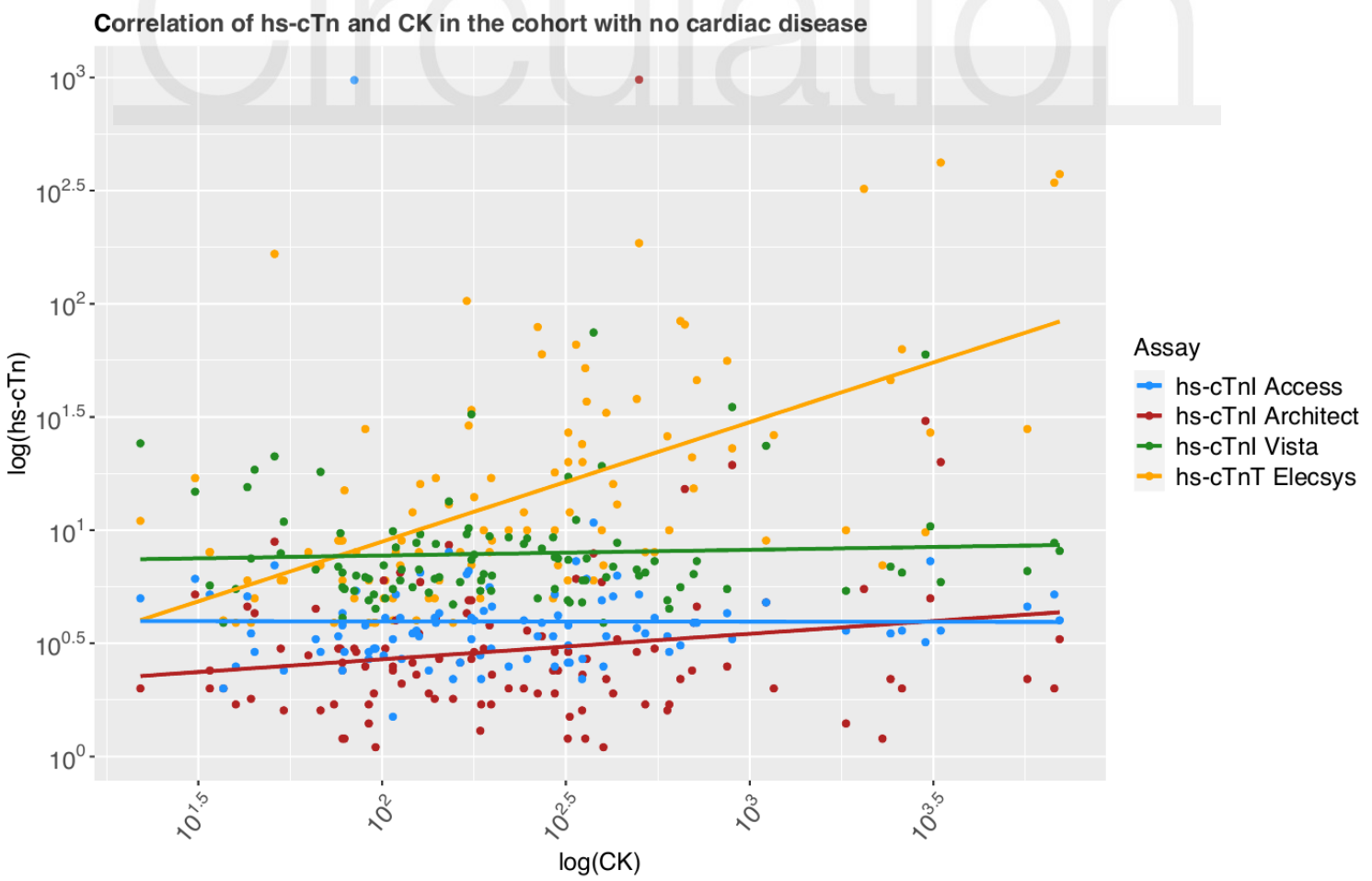

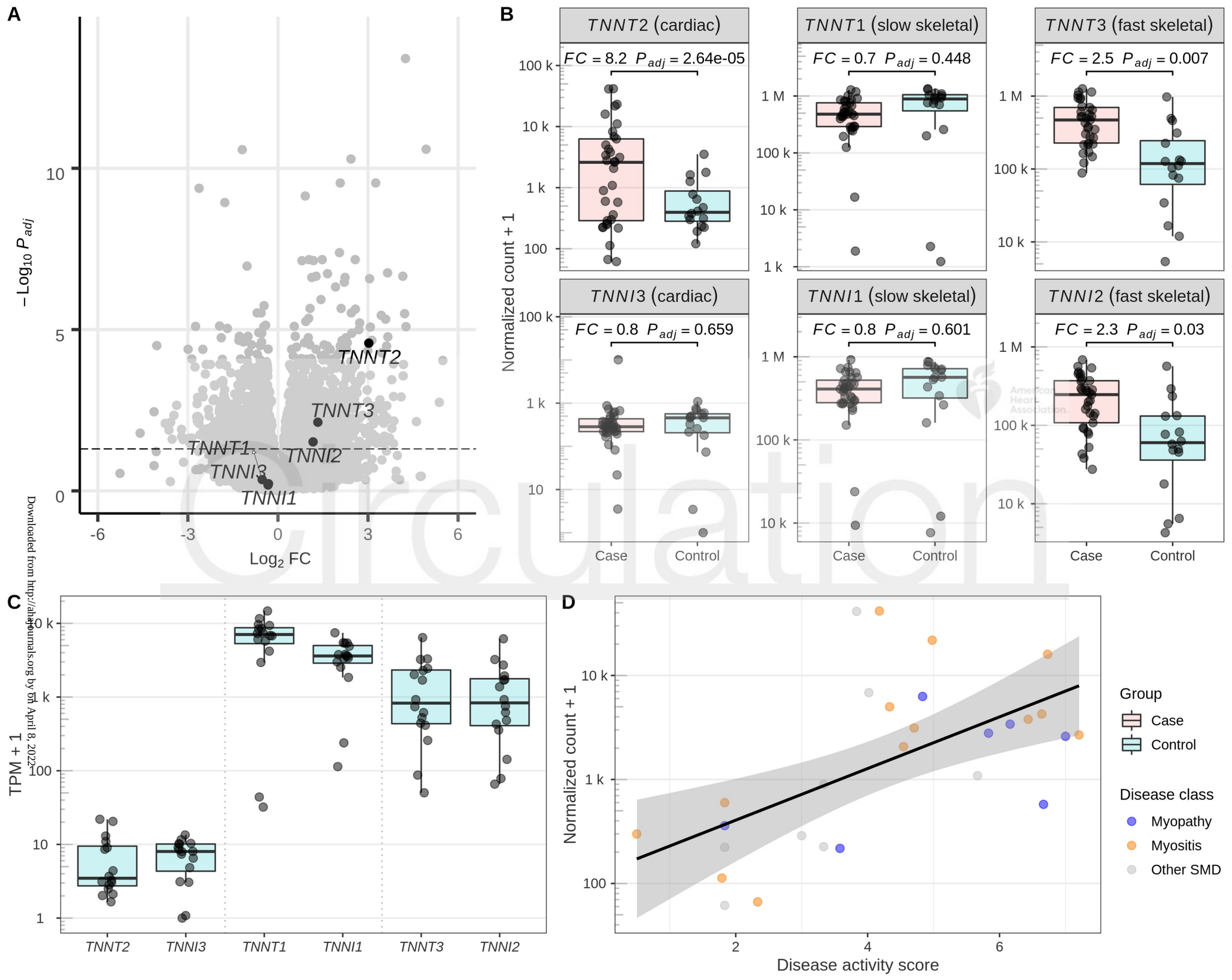

Correlation of hs-cTnT and gene expression in the patients providing muscle biopsies